

Original article

Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells *in vitro*

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Keywords: diabetes mellitus; mesenchymal stem cells; insulin; transdifferentiation

Background Stem cells, which have the ability to differentiate into insulin-producing cells (IPCs), would provide a potentially unlimited source of islet cells for transplantation and alleviate the major limitations of availability and allogeneic rejection. Therefore, the utilization of stem cells is becoming the most promising therapy for diabetes mellitus (DM). Here, we studied the differentiation capacity of the diabetic patient's bone marrow-derived mesenchymal stem cells (MSCs) and tested the feasibility of using MSCs for β -cell replacement.

Methods Bone marrow-derived MSCs were obtained from 10 DM patients (5 type 1 DM and 5 type 2 DM) and induced to IPCs under a three-stage protocol. Representative cell surface antigen expression profiles of MSCs were analysed by flow cytometric analysis. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect multiple genes related to pancreatic β -cell development and function. The identity of the IPCs was illustrated by the analysis of morphology, ditizone staining and immunocytochemistry. Release of insulin by these cells was confirmed by immunoradioassay.

Results Flow cytometric analysis of MSCs at passage 3 showed that these cells expressed high levels of CD29 (98.28%), CD44 (99.56%) and CD106 (98.34%). Typical islet-like cell clusters were observed at the end of the protocol (18 days). Ditizone staining and immunohistochemistry for insulin were both positive. These differentiated cells at stage 2 (10 days) expressed nestin, pancreatic duodenal homeobox-1 (PDX-1), Neurogenin3, Pax4, insulin, glucagon, but at stage 3 (18 days) we observed the high expression of PDX-1, insulin, glucagon. Insulin was secreted by these cells in response to different concentrations of glucose stimulation in a regulated manner ($P < 0.05$).

Conclusions Bone marrow-derived MSCs from DM patients can differentiate into functional IPCs under certain conditions *in vitro*. Using diabetic patient's own bone marrow-derived MSCs as a source of autologous IPCs for β -cell replacement would be feasible.

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METHODS

Subjects

Study subjects comprised 5 type 1 DM patients and 5 type 2 DM patients (6 men and 4 women, mean age (28.5 ± 5.3) years, range 19–42 years). All patients were diagnosed according to ADA criteria and identified without major acute or chronic complications. The duration of diabetes was 2–6 years. The patients were recruited voluntarily from the in-patient service at Qilu Hospital of Shandong University and informed consent was given. The protocol had been approved by the Institutional Review Board of Shandong University.

Study methods

Isolation and culture of bone marrow-derived MSCs

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Diabetes mellitus (DM) results when there is the progressive failure of functional β -cells. The success of Edmonton protocol for pancreatic islet transplantation has sparked new interests in treating DM. Unfortunately, islet transplantation has historically been hampered by immune rejection as well as the scarcity of donor islets.^{1,2} One way to overcome these obstacles and obtain functional glucose-sensitive insulin-producing cells (IPCs) for transplantation is to derive islet cells from stem cells such as embryonic stem (ES) cells, hepatic, ductal, pancreatic or adipose tissue-derived stem cells.³⁻⁷ More significantly, bone marrow-derived mesenchymal stem cells (MSCs) carry the more important implications for possible clinical development, because they are easily accessible for an autograft and routinely collected from adults without the ethical concern inherent to fetal embryonic tissues.⁸⁻¹⁰ Some studies suggested that bone marrow-derived MSCs can differentiate into IPCs both *in vitro* and *in vivo*.^{11,12} But other publications presented conflicting observations.^{13,14} Therefore, we investigated whether the diabetic patient's own bone marrow-derived MSCs contribute to the turnover of autologous IPCs for β -cell replacement.

Under aseptic condition, 5 ml of heparinized bone marrow samples were obtained from the posterior superior iliac crest. Diluted sample with low-glucose Dulbecco's modified Eagle's medium (DMEM, 5.56 mmol/L, Invitrogen Corporation, Grand Island, NY, USA) was layered on top of a density gradient solution (Ficoll-Hypaque, 1.077 g/ml, Haoyang Biological manufacture, CO. Tianjin, China) and centrifuged for 20 minutes at 600 g. Following centrifugation, cells were removed from the plasma/Ficoll-Hypaque interface, and suspended in 5 ml of low glucose DMEM supplemented with 10% fetal calf serum (Hyclone Laboratories, Inc, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Qilu Pharm Inc, Ji'nan, China). After standard nucleated cell counting, cells were plated at a density of 5×10^6 per 25-cm² culture flask, and then incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 72 hours, the non-adherent cells were discarded and the adherent cells were cultured for approximately 20 days. Fresh complete medium was replaced twice a week. Primary cultures were maintained for 18–20 days. Upon reaching near confluence, cells were detached with a solution of 0.25% trypsin (Sigma-Aldrich, Saint Louis, Missouri, USA) and 1 mmol/L ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich, Saint Louis, Missouri, USA) for 3–5 minutes at 37°C. After centrifugation, cells were resuspended with the medium mentioned above, re-plated at a ratio of 1:2 and referred to as first-passage cultures.

Induction of bone marrow-derived MSCs to IPCs

The bone marrow-derived MSCs at passages 3 were induced to IPCs by a three-stage protocol. Stage 1: The cells (1×10^5 /ml) were cultured (37°C, 5% CO₂) for 2 days in serum-free high glucose DMEM (25 mmol/L) containing 0.5 mmol/L β-mercaptoethanol (Invitrogen Corporation, Grand Island, NY, USA). Stage 2: The cells then were cultured in the medium containing 1% non-essential amino acids (Gibico BRL, Life Technologies, UK), 20 ng/ml β-fibroblast growth factor (bFGF, Sigma-Aldrich, Saint Louis, Missouri, USA), 20 ng/ml (EGF, Sigma-Aldrich, Saint Louis, Missouri, USA), 2% B27 (Gibico BRL, Life Technologies, UK), 2 mmol/L L-glutamine (Hyclone Laboratories, Inc, USA) in 6-well plates for 8 days. Stage 3: The cells were cultured for an additional 8 days in new medium containing 10 ng/ml β-cellulin (Sigma-Aldrich, Saint Louis, Missouri, USA), 10 ng/ml activin A (Sigma-Aldrich, Saint Louis, Missouri, USA), 2% B27, 10 mmol/L nicotinamide (Sigma-Aldrich, Saint Louis, Missouri, USA).

Flow cytometric analysis

The bone marrow-derived MSCs at passage 3 were released by trypsinization. The cells were centrifuged at 300 g for 8 minutes, and then were solved in phosphate-buffered saline (PBS) at the concentration of 1×10^6 /ml. The fluorescent labeled direct antibodies (10 μl for each sample) were added and incubated for 30 minutes at room temperature. Labeled cells were thoroughly washed with two volumes of PBS and fixed in flow buffer (1% formaldehyde in PBS). The labeled cells were analyzed on a FACS Caliber (Becton-Dickinson, Franklin Lakes, NJ, USA) by collecting 10 000 events with the Cell Quest software program (Becton-Dickinson, Franklin Lakes, NJ, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from 5×10^6 pre-treated or post-treated MSCs were extracted with TRIzol reagent (Invitrogen Corporation, Grand Island, NY, USA) according to the manufacturer's instructions. Gene expression levels of nestin, pancreatic duodenal homeobox-1 (PDX-1), Neurogenin 3 (Ngn₃), Paired box gene 4 (Pax4), insulin, and glucagon were determined by RT-PCR. Reverse transcription was carried out using 1 μg of total RNA, 20 mmol/L dNTP (Invitrogen Corporation, Grand Island, NY, USA), 1 mmol/L random hexamers (Invitrogen Corporation, Grand Island, NY, USA), and 100 U reverse transcriptase (Invitrogen Corporation, Grand Island, NY, USA) in a total volume of 20 μl. PCR was carried out using 2 μl cDNA in a total volume of 20 μl. The primer sequences for RT-PCR were showed in Table.

Diphenylthiocarbazon staining

Ditizone (10 mg, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was completely dissolved in 10 ml of dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, Missouri, USA) and was stored at -20°C. The working solution (pH 7.8) was prepared immediately prior to use by diluting the stock solution 1:10 in PBS. For each dish, 2 ml of the ditizone solution were added and incubated for 30 minutes at 37°C.

Immunocytochemistry

The differentiated cells adherent to slides were fixed with 40 g/L para-formaldehyde. After washed with PBS for 3 times, the slides were incubated with a polyclonal rabbit anti-human insulin (1:100, ZYMED Laboratories, San Francisco, California, USA) or polyclonal rabbit anti-human glucagon (1 : 200, ZYMED Laboratories, San Francisco, California, USA) at 4°C overnight. Immuno-reactive cells

Table. Primer sequences for RT-PCR

Genes	Primers		Conditions
	Sense	Antisense	
Nestin	gagggcaagtgtgaagcag	gctctcatcccactctc	30 cycles of 94°C, 45 sec; 58°C, 45 sec; 72°C, 45 sec; a final extension at 72°C for 10 min
PDX-1	gagctggctgtcatgttga	agtgggtgaagcccctcag	30 cycles of 94°C, 30 sec; 57°C, 30 sec; 72°C, 30 sec; a final extension at 72°C for 10 min
Ngn3	tcgctctcatcgtctctc	ccaactcgtcttaggcc	30 cycles of 94°C, 60 sec; 56°C, 45 sec; 72°C, 45 sec; a final extension at 72°C for 10 min
Pax4	aagggtgagtgtccagcc	aagatagtcggattccgg	30 cycles of 94°C, 30 sec; 58°C, 30 sec; 72°C, 30 sec; a final extension at 72°C for 10 min
Insulin	ctacctagtgtcggggaac	cacaatgccacgcttctg	30 cycles of 94°C, 30 sec; 61°C, 30 sec; 72°C, 30 sec; a final extension at 72°C for 10 min
Glucagon	aggcagaccactcagtgat	cggccaagtcttcaacaat	30 cycles of 94°C, 45 sec; 55°C, 45 sec; 72°C, 45 sec; a final extension at 72°C for 10 min

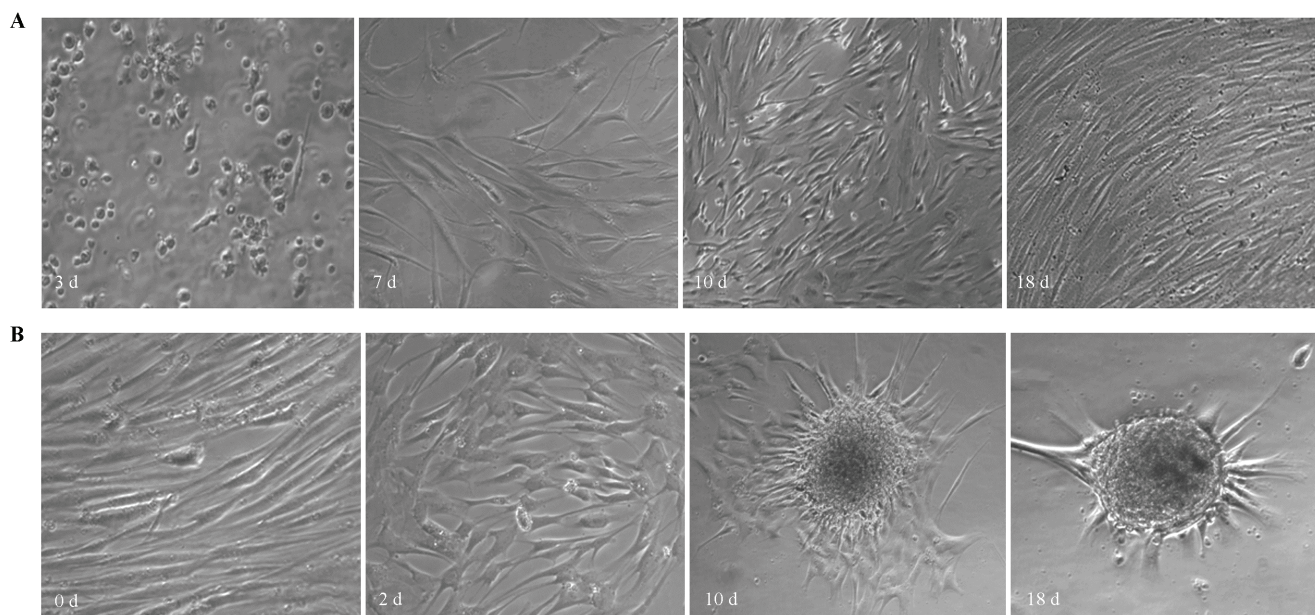


Fig. 1. Isolation and characterization of bone marrow-derived MSCs. **A:** Morphological changes of undifferentiated bone marrow-derived MSCs. Cells from diabetic patients were plated and cultured to obtain the adherent mature MSCs on day 18 (Original magnification $\times 10$). **B:** Morphological changes of bone marrow-derived MSCs differentiation. MSCs were induced to IPCs according to differentiation protocol by culturing in the presence of 25 mmol/L glucose concentration from day 0 to day 18 (Original magnification $\times 10$).

were visualized using the Vectastain Elite ABC Kit (Vector Labs, USA) with 3'3 diaminobenzidine tetrachloride (DAB, Boehringer- Mannheim, Inc, Germany).

Enzyme immunoassay

The differentiated cells were pre-incubated for 1 hour in glucose-free Krebs-Ringer bicarbonate (KRB), and incubated with KRB containing 5.56 mmol/L, 16.7 mmol/L or 25 mmol/L of glucose for an additional 1 hour, respectively. The KRB media were collected and frozen at -80°C until assay. Insulin assay was performed by enzyme-immunoassay (Human Insulin ELISA kit, Linco Research, Inc. St. Charles, MO, USA) according to the manufacturer's instruction.

Statistical analysis

The results were expressed as mean \pm standard deviation (SD). The statistical significance of differences was assessed by the student's *t* test. In all comparisons, a value of $P < 0.05$ was considered statistically significant.

RESULTS

Phenotype characteristics of expanded undifferentiated bone marrow-derived MSCs

After plastic adherence selection, bone marrow-derived MSCs were cultured over three passages. Flow cytometric analysis of the MSCs at the passage 3 showed that these cells were negative for CD34 (1.43%), CD45 (2.39%) and CD14 (6.3%). They expressed high levels of CD29 (98.28%), CD44 (99.56%) and CD106 (98.34%). These results indicated that relatively purified bone marrow-derived MSCs were isolated.

Morphological changes of bone marrow-derived MSCs differentiation

Under a inverted microscope, undifferentiated MSCs were typical of adherent spindle and fibrocyte-like (Fig. 1A). However, under differentiation, these spindle-like cells growing as adherent cultures can be induced to grow in an attachment-independent fashion, forming spherical type with confluence (Fig. 1B). The morphology of cell clusters look like islet-like structure.

Gene expression of bone marrow-derived IPCs

To determine whether the bone marrow-derived MSCs had undergone pancreatic differentiation, gene expression profiles for pancreatic cell differentiation markers and hormones were assessed using RT-PCR. As illustrated in Fig. 2, no expression of nestin, PDX-1, Ngn3, Pax4, insulin and glucagon was detected in undifferentiated MSCs. As a positive control, the expression of GAPDH was detected indicating our experimental system being intact. When the cells were induced for the first 8 days (stage 2), all of the six genes were expressed with low level of insulin and glucagon. After differentiation for another 8 days (stage 3), the cells expressed no nestin, Ngn3 and Pax4 but high level of PDX-1, insulin, and glucagons.

Insulin detection in islet-like structures

To verify the insulin expression in the islet-like structure, ditizone which specifically stains insulin granules present in β -cell was used to evaluate the islet number and their sizes. As shown in Fig. 3A, most of the cells were positive for ditizone staining ($(82 \pm 15)\%$, $n=10$). There were 3.35 ± 0.92 islets in each low power field of vision and

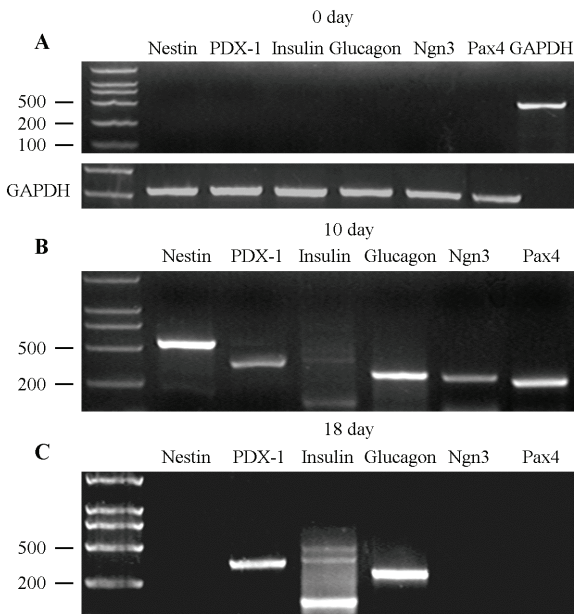


Fig. 2. mRNA expression of differentiation-related genes in different stages. RT-PCR was performed to detect genes related to pancreatic β -cell development and insulin production. **A:** Pancreatic islet-specific transcripts—nestin, PDX-1, Ngn3, Pax4, insulin and glucagon were not expressed under uninduced conditions. GAPDH mRNA expression was detected as the PCR positive control and input template control. **B:** These transcripts were all expressed on the day 10 under differentiation conditions. **C:** On day 18, bone marrow-derived IPCs can only express PDX-1, insulin and glucagon. PDX-1: pancreatic duodenal homeobox-1; Ngn3: neurogenin 3; Pax4: paired box gene 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

the average diameter of the islet was about 120–200 μm .

Protein expression of bone marrow-derived IPCs

To confirm the insulin and glucagon expression of the bone marrow-derived IPCs at the protein level, the undifferentiated and differentiated cells were assessed by immunocytochemistry. All of the undifferentiated cells were negative for insulin and glucagon. In contrast, some differentiated cells in the islet were positive for insulin

while others expressed glucagon (positive for insulin and glucagon was $68\% \pm 14\%$ and $55\% \pm 16\%$, respectively, $n=10$, Fig. 3B and C). However, some cells expressed neither insulin nor glucagon, indicating not all bone marrow-derived MSCs were differentiated under these conditions.

Insulin release in response to glucose stimulation

To further determine whether the bone marrow-derived IPCs were responsive to a glucose challenge, insulin release from undifferentiated and differentiated cells was measured using an ultrasensitive human insulin ELISA kit. The differentiated cells could release insulin (46.5 ± 3.6 $\mu\text{IU/ml}$) in a low concentration of glucose (5.56 mmol/L) and release approximately threefold insulin (113.7 ± 9.2 $\mu\text{IU/ml}$) under glucose challenge (25 mmol/L) ($P < 0.05$). In contrast, undifferentiated bone marrow-derived MSCs showed no significant release of insulin in the presence or absence of glucose challenge. (Fig. 4)

DISCUSSION

Transplantation of pancreatic islet cells and utilization of stem cells as a potential cure for DM have become the subjects of intense interest and activity over the past several years.^{1,15,16} However, some obstacles, such as limited supply of human islet tissue, immune rejection, and issues of ethics, still remain. Bone marrow has been known for years as a safe and abundant source for large quantities of adult stem cells. Hess et al¹⁷ tested the capacity of transplanted bone marrow-derived stem cells to initiate endogenous pancreatic tissue regeneration in diabetic mice. Tang et al¹¹ also reported the same results. Yet other reports demonstrated that such events only occur at very low frequency, and the apparent plasticity has been shown in one case to be explained by cellular fusion.^{18,19} However, most of these studies mentioned above were based on animal models and little is known about the differentiation of bone marrow-derived stem cells into IPCs from humans, especially diabetic objects. Therefore, in this study we identified, characterized, and propagated

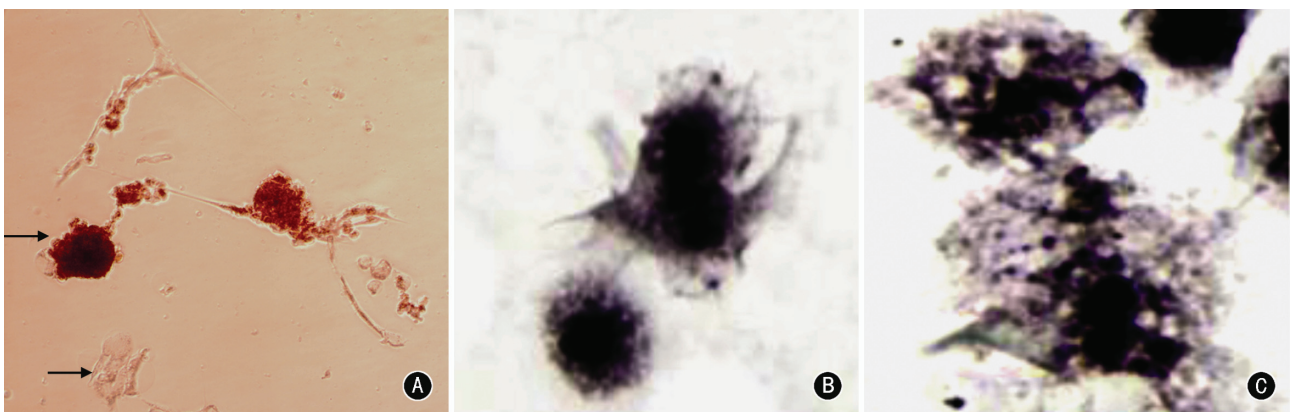


Fig. 3. Ditione staining and immunohistochemistry for insulin and glucagon. Bone marrow-derived IPCs distinctly stained crimson red by ditizone are apparent, with cells assembled as masses (A, original magnification $\times 10$). Most of the clusters were positive for insulin (B) and glucagon (C) (Original magnification $\times 40$).

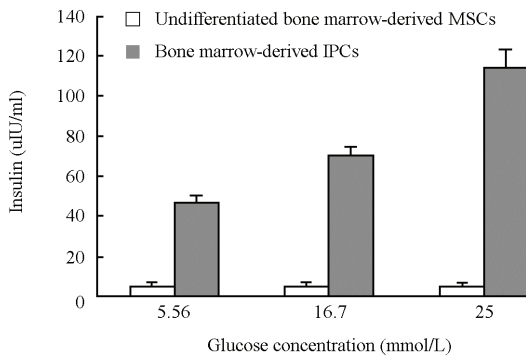


Fig. 4. Insulin release in response to glucose challenge. The statistical significance ($P < 0.05$) of the data was evaluated by comparing bone marrow-derived IPCs treated with different glucose challenge.

bone marrow-derived MSCs from diabetic patients and induced them to IPCs under certain condition *in vitro*. This provided the direct evidence that the differentiation capacity of bone marrow-derived MSCs in diabetic patients was intact. The induced IPCs were morphologically similar to pancreatic islet-like cells. More importantly, they not only produced insulin but also could secrete insulin in response to different concentration of glucose stimulation in a regulated manner. So we considered that bone marrow-derived MSCs from diabetic patient's as a source of autologous IPCs for β -cell replacement, which could avoid immune rejection and ethical issue, is feasible.

In our experiment, the phenotype of bone marrow-derived stem cells is negative for CD34, CD45 and CD14, which indicated they were unlikely to be hematopoietic stem cells. On the contrary, CD29, CD44 and CD106 are positive indicating that the bone marrow-derived stem cells capable of generating IPCs might be bone marrow-derived MSCs. This is consistent with other reports.^{20,21} Nestin expression only appeared in stage 2 cells but not in stage 1 or 3 cells, which implied that marrow-derived MSCs might differentiate into IPCs through an intermediate neurocyte stage. However, another study reported that nestin was expressed both in undifferentiated and differentiated cells.¹¹ The discrepancy might originate from species differences or the different culture protocol. We also observed that the time of primary culture and transfer culture were different with previous reports.^{20,22} Only the difference of experimental condition could explain the results. Thus, in this paper, we would like to emphasize the contribution of bone marrow-derived MSCs from diabetic patients to differentiate into IPCs under the experimental condition described here.

Different protocols have been tried to induce bone marrow-derived MSCs to differentiate into IPCs *in vitro*. High glucose concentration was considered as a potent inducer for pancreatic islet differentiation.¹¹ In our primary experiment, high glucose alone could not induce bone marrow-derived MSCs to IPCs. Nicotinamide,

which is an effective inducer, was used to preserve islet viability and function through poly ADP-ribose polymerase (PARP).²³ Activin A is a member of the transforming growth factor- β (TGF- β) superfamily which regulated neogenesis of β -cells *in vivo*.²⁴ Furthermore, β -cellulin played an important role in regulating growth and/or differentiation of pancreatic endocrine precursor cells.^{24,25} Thus, in our experimental system, we observed that a combination of nicotinamide, activin A and β -cellulin in high glucose medium effectively promotes bone marrow-derived MSCs differentiation.

Even under the above conditions, only some of the bone marrow-derived MSCs could differentiate into IPCs. Some cells expressed glucagons, which might represent β -cells while others expressed neither insulin nor glucagon. The efficiency of differentiation was relatively low because of the obscurity of the cellular mechanism underlying β -cell differentiation which could account for the conflicting and different results in the area.²⁶

Although the destruction of β -cells was typically considered as the cause of type 1 DM, more and more findings indicated the dysfunction of β -cells also participated in the development of type 2 DM and insulin is usually used in the treatment of type 2 DM.²⁷ Based on these evidences, the cell transplantation might also be effective to the treatment of type 2 DM. Due to this reason, we also isolated the bone marrow-derived MSCs from type 2 diabetic patients and induced them to IPCs *in vitro*. Our results showed there was no difference in the capacity of the bone marrow-derived MSCs between the two types of diabetic patients to differentiate into IPCs *in vitro*.

In summary, the data presented here demonstrate that the bone marrow-derived MSCs from both type 1 and type 2 diabetic patients can differentiate into IPCs under appropriate conditions *in vitro*. These results provide the direct evidence for the feasibility of autologous transplantation utilizing bone marrow-derived MSCs in the therapy of DM.

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